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INVITED REVIEW

Development and potential of genetically engineered oilseeds

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Abstract

Oilseed crops are major sources of oils for human nutrition, and an increasing proportion is also being utilized for industrial purposes. Recent advances in our understanding of the basic biochemistry of seed oil biosynthesis, coupled with identification of genes for oilseed modification, have set the stage for the genetic engineering of oilseed crops that produce 'designer' plant seed oils tailored for specific applications. In this review we provide an overview of seed oil biosynthesis and highlight the enzymatic steps that have already been targeted for genetic manipulation, with the end goal of producing seed oils containing desired amounts of fatty acid components. Furthermore, we describe the identification of genes from various wild plant species that are capable of producing structurally diverse fatty acids, and how these advances open the door to the production of entirely novel oils in conventional oilseed crops. Transgenic oilseeds producing high amounts of these novel fatty acids represent renewable sources of raw materials that may compete with, and eventually replace, some petrochemicals that are derived from non-renewable crude oil.

Keywords: fatty acid, genetic engineering, oil biosynthesis, oilseed, wax

Introduction

Oilseed crops are major agricultural commodities, with 320 million metric tons produced worldwide in 2002, at a value of over 60 billion US dollars. All of the major oilseed crops have been modified by genetic engineering (GE), and the majority of the world's largest oilseed producers have openly embraced this revolutionary biotechnology.

While the vast majority of genetically modified (GM) oilseed crops produced to date contain herbicide tolerance or insect resistance, the greatest potential of oilseed crops probably lies in the genetic manipulation of fatty acid content to improve either the nutritional, physical, chemical or industrial properties of oils. For instance, while approximately 85% of vegetable oils are currently used in food and cooking applications (Broun *et al.*, 1999), identification of genes involved in the synthesis of industrially important fatty acids opens the door to production of so-called oleochemicals in oilseed crops. These types of plant oils represent a diverse array of renewable resources that could potentially supplant traditional petrochemicals derived from crude oil.

Adoption of GM oilseeds

All of the major oilseed crops (Table 1) have been successfully transformed using GE technology, and GM varieties have been adopted to various extents by most of the world's largest agricultural producers, including the USA, Argentina, Canada, China and, recently, India. For some crops, GM varieties may represent the majority of total planted acreage. In the USA, for example, transgenic soybeans and cotton currently account for over 70% of the planted acreage of each crop (Fernandez-Cornejo and McBride, 2002). Several other countries, such as Canada, have also embraced GE technology, as evidenced by the fact that over 60% of canola (a variety of rapeseed with low erucic acid) in Canada is GM (James, 2001). China is also pursuing the development of GE technology aggressively, currently studying more than 50 plant species and more than 120 candidate genes (Huang *et al.*, 2002).

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Table 1. Major oilseed crops and producers (adapted from the USDA Foreign Agricultural Service, World Agricultural Production Archives (http://www.fas.usda.gov/wap_arc.html). Data represent averages from the 2000/01 to 2002/03 growing seasons

Crop	Area[a]	Production[b]	Top three producers[c]		
Soybean	78.84	185.68	USA (76.25)	Brazil (44.83)	Argentina (30.93)
Cottonseed	31.13	34.33	China (8.79)	USA (6.07)	India (4.80)
Rapeseed	23.58	35.37	China (11.09)	EU (9.85)	Canada (5.47)
Peanut	22.49	31.83	China (14.56)	India (6.17)	USA (1.64)
Sunflower	19.75	22.25	FSU-12[d] (6.68)	EU (3.62)	Argentina (3.53)
Total oilseeds	175.80	310.05	USA (86.22)	China (52.00)	Brazil (46.49)

[a]Million hectares, worldwide.
[b]Million metric tons, worldwide.
[c]Country/region (million metric tons).
[d]Twelve states representing the former Soviet Union.

Second-generation GM oilseeds

While the first generation of transgenic oilseeds offered advantages to farmers in the production phase of agriculture, the second generation of GM oilseeds contains output traits that meet the needs of consumers and industry. All oilseeds contain storage reserves in the form of lipids, proteins and carbohydrates, and each of these constituents is currently a target for GE. Examples of some of the current GE goals are increased nutritional quality and digestibility of seed proteins (Herbers and Sonnewald, 1999), modification of carbohydrate content for improved consistency and taste (Heyer *et al.*, 1999), and alteration of lipid contents to improve nutritional, physical or chemical properties of seed oils (Broun *et al.*, 1999; Murphy, 1999; Thelen and Ohlrogge, 2002). An excellent example of how seed companies have responded to consumer demands for improved quality and nutrition is the development of canola. With the goal of developing a low-erucic acid rapeseed with highly unsaturated oil for improved nutritional quality, Canadian scientists in the 1970s used traditional breeding techniques to produce this high oleic acid variety of rapeseed. Canola contains $<30\ \mu moles\ g^{-1}$ glucosinolates, and has an oil profile that is high in monounsaturates, moderate in polyunsaturates and low in saturates. Today, canola accounts for two-thirds of Canada's oilseed production, with an estimated value of $2 billion (Lavers, 2002).

Although traditional breeding methods have been used successfully to alter the fatty acid composition of oils, GE provides a more rapid and direct method for manipulating fatty acid composition and can greatly expand the types of fatty acids produced in oil seeds. Recent advances in our understanding of the biochemical, cellular and molecular mechanisms of seed oil biogenesis, coupled with the cloning of genes involved in this process, have facilitated the production of novel 'designer oils' in oilseed crops. These advances and the types of GM oilseeds with nutritional and/or industrial uses are summarized in this review. An exhaustive list of all approved GM crops, including detailed descriptions of their genetic modifications, is available on-line from AGBIOS at http://www.agbios.com/dbase.php?action=ShowForm. A list of all GM plants at various stages of field testing and development within the USA is maintained by the USDA Animal and Plant Health Inspection Service (http://www.aphis.usda.gov/index.html).

Overview of storage oil biosynthesis: potential targets for GE

Seed storage oil, which can comprise from 1 to 75% of dry seed weight, serves as the primary carbon and energy reserve for early seedling development. Most storage oils are composed of triacylglycerols (TAGs) that are synthesized and deposited during a brief period of seed development. Unlike the fatty acid composition of cellular membranes, which is restricted to a small set of conserved fatty acids, the fatty acid composition of TAGs can be highly divergent, with over 300 fatty acid structures observed in nature (Smith, 1970). These storage oil fatty acids may vary in chain length, number and position of double or triple bonds, and may have additional functional groups, such as hydroxy or epoxy groups. The varied functional groups may also serve as targets for further chemical modification, making these types of fatty acids potential precursors for industrial processes. Notably, the majority of domesticated oilseed crops accumulate common fatty acids that are also present in the cellular membranes of all plants, while many plant species with limited agronomic traits produce 'exotic' or 'unusual' fatty acids. Therefore, a major goal for GE is to engineer the metabolic pathways for production of exotic fatty acids into more traditional oilseed crops.

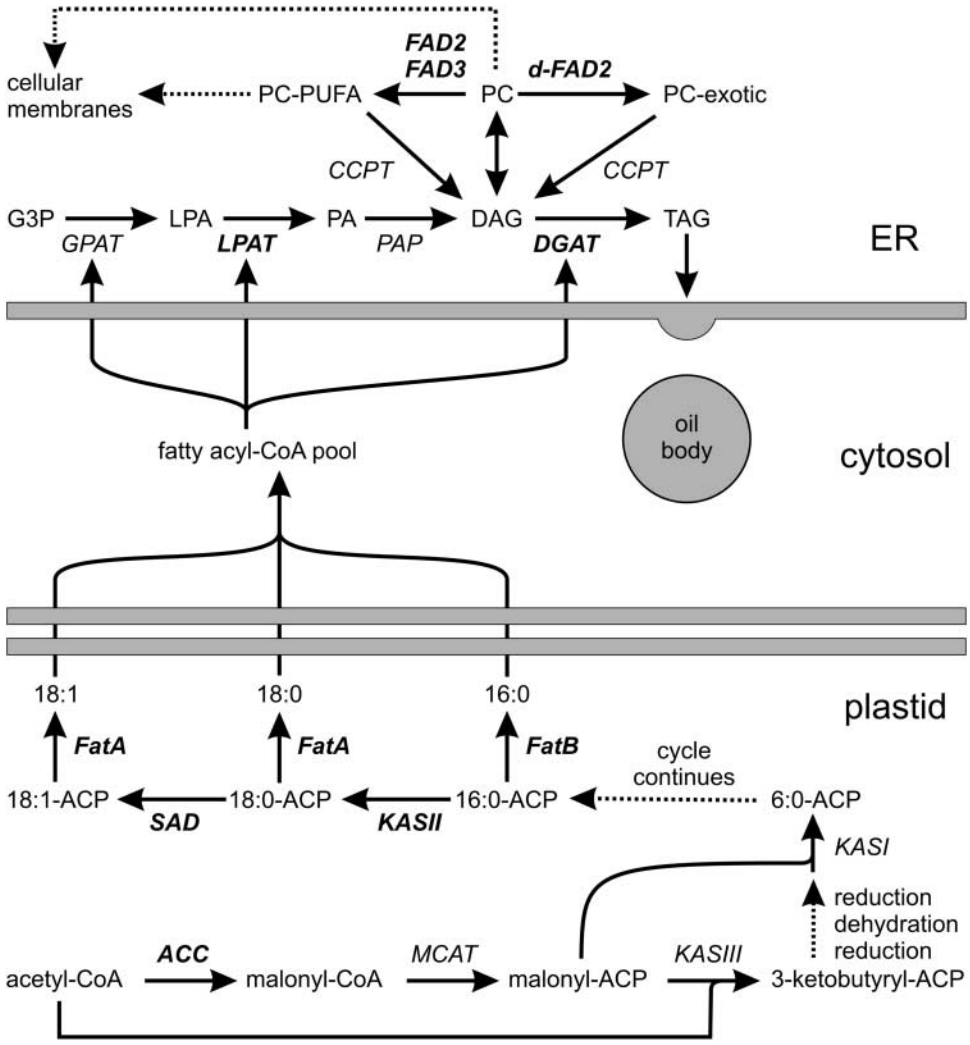

Figure 1. Biosynthesis of storage oils in developing seeds. Shown are the salient features of fatty acid biosynthesis in the plastid, incorporation of fatty acids into glycerolipids in the endoplasmic reticulum (ER) and modification of fatty acids by membrane-bound enzymes prior to synthesis and accumulation of triacylglyerols in cytosolic oil bodies. Enzymes (italics) in bold lettering have been manipulated through genetic engineering (see text for details). Enzyme abbreviations are: acetyl-CoA carboxylase (*ACC*), malonyl-CoA:ACP transferase (*MCAT*), 3-ketoacyl-ACP synthases (*KASI*, *II* or *III*), acyl-ACP thioesterases (*FatA* or *FatB*), stearoyl-ACP desaturase (*SAD*), glycerol 3-phosphate acyltransferase (*GPAT*), lysophosphatidic acid acyltransferase (*LPAT*), phosphatidic acid phosphatase (*PAP*), diacylglycerol acyltransferase (*DGAT*), CDP-choline:DAG cholinephosphotransferase (*CCPT*), fatty acid desaturase 2 (*FAD2*), fatty acid desaturase 3 (*FAD3*), diverged FAD2 (*d-FAD2*). Glycerolipid abbreviations are: glycerol-3-phosphate (G3P), lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerol (DAG), triacylglycerol (TAG), phosphatidylcholine (PC), phosphatidylcholine-containing common polyunsaturated fatty acids present in most traditional crops (PC-PUFA), phosphatidylcholine-containing uncommon fatty acids present in some exotic plant species (PC-exotic). Other abbreviations are: coenzyme A (CoA) and acyl carrier protein (ACP). More detailed information regarding the pathways and the associated enzymes involved in seed storage oil biosynthesis can be obtained elsewhere (Buchanan *et al*., 2000).

The biochemical processes involved in biosynthesis of seed storage oils are well established, and involve metabolic pathways located in several different subcellular organelles. Figure 1 highlights these organellar compartments and provides a general description of their resident metabolic pathways and enzymes. The most attractive enzyme targets for biotechnological manipulation are highlighted to serve as a point of reference for subsequent sections in this review that describe GE manipulation in greater detail.

Fatty acid biosynthesis occurs in the plastids of developing seeds, with the enzyme acetyl-CoA carboxylase (ACC) catalysing the first committed

step in the pathway. All of the fatty acid constituents of storage oils are derived from the pool of acetyl-CoA present in the plastid. ACC activates acetyl-CoA to malonyl-CoA by addition of a carboxyl group, and this carboxyl group subsequently serves as a leaving group to drive fatty acid synthesis. The malonyl group is then transferred from coenzyme A (CoA) to a small protein termed the acyl-carrier protein (ACP), which serves as the carrier for the growing fatty acid chain. Malonyl-ACP is reacted with a second acetyl-CoA by a condensing enzyme, ketoacyl-ACP synthase III (KASIII), resulting in formation of a four-carbon side-chain (the carboxyl group of malonyl CoA is released in the process). The resulting four-carbon compound (3-ketobutyryl-ACP) then undergoes a sequence of three reactions – reduction, dehydration and reduction again – and is finally condensed with another malonyl-ACP, resulting in a six-carbon side-chain. The process is then repeated, two carbon units at a time being added, until a saturated fatty acid chain of either 16 (16:0) or 18 (18:0) carbons in length is produced.[1] In some plants, the elongation of the nascent fatty acid is prematurely terminated by a specific thioesterase enzyme that is part of the FatB class of thioesterases. FatB thioesterases cleave the growing fatty acyl side-chain from ACP, resulting in accumulation of short- to medium-chain fatty acids in seed oil that range in length from 8:0 to 14:0 (Pollard *et al.*, 1991; Davies, 1993).

Three different isoforms of the KAS enzyme are involved in fatty acid synthesis (elongation) in plastids (Ohlrogge and Browse, 1995). As mentioned above, one KAS isoform, referred to as KASIII, catalyses the initial condensation reaction between malonyl-ACP and acetyl-CoA to produce 3-ketobutyryl-ACP. KASI, on the other hand, catalyses subsequent condensations between the growing acyl side-chain and additional malonyl-ACPs to produce 6:0–16:0 fatty acids. Finally, KASII completes the synthesis of an 18:0 fatty acid side-chain by catalysing the condensation between malonyl-ACP and 16:0-ACP. The KASII isoform, therefore, plays an important role in determining the ratio of 16:0 and 18:0 fatty acids in the oil, and is considered an attractive target for GE.

Overall, there are several different fates for 16:0-ACP in the plastid, and each of the various pathways available to this metabolite can have a major influence on overall fatty acid composition of the seed oil. In species such as palm, the 16:0 fatty acyl side-chain (referred to as a palmitoyl group) is cleaved from ACP by a FatB thioesterase, resulting in seed oils that are high in palmitic acid. Palmitoyl-ACP may also be elongated to an 18:0 fatty acyl side-chain (referred to as stearoyl-ACP) by KASII, which can subsequently be cleaved by another class of thioesterases referred to as FatA-type thioesterases. Alternatively, stearoyl-ACP can be desaturated by a soluble stearoyl-ACP desaturase present in the plastid stroma to produce oleoyl-ACP (18:1Δ^{9cis}), which is also cleaved by a FatA thioesterase. Since oleic acid can be further desaturated by enzymes located in the endoplasmic reticulum (ER) (see below), the relative activities of FatB thioesterase, KASII, FatA thioesterase and stearoyl-ACP desaturase play an important role in determining the final amounts of saturated and unsaturated fatty acids in seed storage oils.

After release from ACP by varied thioesterases and then ligation to CoA at the plastid outer membrane (Schnurr *et al.*, 2002), 16:0, 18:0 and 18:1Δ^{9cis} fatty acyl-CoAs exit the plastid and are used for the synthesis of glycerolipids in the ER. The traditional glycerolipid biosynthetic pathway features consecutive transfer of fatty acids from fatty acyl-CoAs to the *sn*-1 and *sn*-2 positions of glycerol-3-phosphate, resulting in phosphatidic acid (PA). These fatty acyl additions are catalysed by glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAT), respectively (Fig. 1). Removal of the phosphate head group from the *sn*-3 position of PA results in production of diacylglycerol (DAG), and subsequent acylation of the *sn*-3 position of DAG, catalysed by the enzyme diacylglycerol acyltransferase (DGAT), produces the triacylglycerols (TAGs) that accumulate in seed storage oil. A second pathway for TAG synthesis is catalysed by the enzyme phospholipid:diacylglycerol acyltransferase (PDAT), which transfers a fatty acyl-group directly from phosphatidylcholine to DAG without an acyl-CoA intermediate (Stahl *et al.*, 2004).

TAGs are hydrophobic, and thus accumulate in the fatty acyl portion of the ER lipid bilayer. The high amount of TAG eventually results in formation of a 'bulge' in the bilayer and then the pinching-off of an oil droplet, which is surrounded by a phospholipid monolayer. These released TAG storage organelles or oil bodies are typically about one micrometer in diameter and coated with abundant structural proteins, termed oleosins (Huang, 1992, 1996; Murphy, 1993). Due to their low density, oil bodies and their associated membrane proteins may be isolated to high purity by low-speed centrifugation of seed homogenates. This relatively simple separation method for oil bodies has facilitated the development of GE strategies geared towards protein purification (Parmenter *et al.*, 1995), whereby a protein of interest can

[1]Fatty acid nomenclature indicates the number of carbon atoms in the backbone, number of double bonds, and position and configuration of double bonds counted from the carboxyl end of the fatty acid. For example, 18:0 (stearic acid) indicates a fatty acid 18 carbon atoms in length with zero (saturated) double bonds; 18:1Δ^{9cis} (oleic acid) indicates a fatty acid 18 carbons in length with one *cis*-type double bond (unsaturated) at position 9 from the carboxyl end of the fatty acid.

be fused to the oleosin protein, expressed in high amounts in developing seeds, and rapidly purified by isolation of the oil bodies. Unfortunately, this technology has yet to prove cost effective because of apparent difficulties in purifying (cleaving) the protein of interest from oleosin.

The biosynthetic pathway of TAGs is not a simple linear process, providing ample opportunity for modification of the fatty acids that eventually accumulate in them. For example, DAG is readily interconverted to phosphatidylcholine, which serves as a substrate for a variety of ER lipid-modifying enzymes, including fatty acid desaturase 2 (FAD2) and FAD3. These two integral membrane-bound enzymes sequentially introduce double bonds into the fatty acyl side-chains of phosphatidylcholine by converting oleic acid ($18{:}1\Delta^{9cis}$) to linoleic acid ($18{:}2\Delta^{9cis,12cis}$), and linoleic acid into linolenic acid ($18{:}3\Delta^{9cis,12cis,15cis}$), respectively. It is the relative activities of FAD2 and FAD3 that determine the ratios of polyunsaturated fatty acids in all major vegetable oils. Thus, in recent years, the genes encoding these enzymes (particularly FAD2) have been obvious targets for GE strategies geared towards the manipulation of polyunsaturated fatty acid content in seed oils.

Many of the enzymes responsible for synthesis of exotic fatty acids in non-traditional crop plants also have been identified recently, and sequence analyses have revealed that they generally fall into two main groups. The first group represents diverged forms of the acyl-ACP desaturases present in the plastid, and these enzymes typically catalyse the synthesis of unusual monoenoic fatty acids (Shanklin and Cahoon, 1998). The second group represents diverged forms of the FAD2 enzyme in the ER, which exhibit a high degree of catalytic plasticity, capable of introducing hydroxy, epoxy, acetylenic and conjugated double bonds into fatty acids. The ectopic expression of these two groups of enzymes in traditional oilseed crop plants is being pursued aggressively.

Improving nutritional and physical characteristics of edible oils

Plant oils are generally considered healthy alternatives to fats derived from animals. However, plant oils, like most animal fats, may not have the specific physical characteristics required for certain food or cooking applications and, therefore, must be modified prior to use (Gunstone, 1998). For example, vegetable oils containing polyunsaturated fatty acids (Table 2) often require partial hydrogenation to achieve the firmness and consistency required for use in baked goods and margarines. Chemical hydrogenation modifies polyunsaturated fatty acids in several ways, including complete reduction of double bonds into single bonds, conversion of *cis* double bonds into *trans* double bonds, and double bond migration. Collectively, these modifications allow the fatty acids in the oil to pack more closely together, resulting in formation of semi-solid fats at room temperature. The reduction of polyunsaturated fatty acid content by hydrogenation also improves shelf-life and stability of oils in high-temperature cooking applications, since polyunsaturated fatty acids are readily oxidized to form compounds associated with rancidity and off-flavours. While the hydrogenation process may impart these desirable physical textures and/or improve the oxidative stability of oils, the *trans* fatty acids formed during this process are undesirable because they have recently been associated with negative health effects (Stender and Dyerberg, 2004).

Since the melting temperature and oxidative stability of oils are determined primarily by fatty acid composition, a major goal of plant biotechnologists has been to manipulate the ratios of saturated and unsaturated fatty acid content to improve the performance of plant oils in food and cooking applications, and thus avoid the need for chemical hydrogenation. More specifically, a primary objective of genetic engineers has been to increase the amounts of saturated and monounsaturated fatty acids in oils and reduce the amounts of polyunsaturated fatty acids. Overall, manipulation of fatty acid ratios in plant oils has been one of the most successful GE endeavours to date, with a number of different types of oils produced that contain desirable ratios of fatty acid components.

Table 2. Major vegetable oils and their respective fatty acid compositions (adapted from the USDA Foreign Agricultural Service, Major Oilseeds: World Supply and Distribution, Table dated 11 April 2005 (http://www.fas.usda.gov/psd/complete_tables/OIL-table1-4.htm) and Bockisch, 1998)

		Approximate fatty acid composition (%)				
Oil	Production[a]	16:0[b]	18:0	18:1	18:2	18:3
Soybean	30.35	10	4	21	56	8
Palm	27.28	38	5	42	8	0
Canola	11.93	4	2	56	21	10
Sunflower	8.17	7	5	24	63	0
Peanut	4.52	9	4	48	22	0
Cotton	3.53	24	4	20	47	0
Palm kernel[c]	3.30	8	3	13	2	0
Coconut[c]	3.22	8	3	6	2	0
Olive	2.39	10	2	65	10	0

[a]Worldwide production (million metric tons) in 2002–2003.
[b]Abbreviations are: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid.
[c]Oil contains over 60% short- and medium-chain fatty acids (8:0–14:0, mainly 12:0).

High oleic acid oils

As mentioned, canola oil, which is high in oleic acid, low in total saturates and has a modest amount of polyunsaturates, was developed through traditional breeding methods. In GE-based strategies, the main approach for producing high oleic acid oils is suppression of the FAD2 enzyme, which normally converts oleic acid ($18{:}1\Delta^{9cis}$) into linoleic acid ($18{:}2\Delta^{9cis,12cis}$). Disruption of FAD2 activity should result in accumulation of the monounsaturated fatty acid substrate, oleic acid, and reduction of the polyunsaturated fatty acid product, linoleic acid. Indeed, suppression of FAD2 activity in transgenic soybean increases oleic acid content up to 86%, with a concomitant reduction in linoleic acid to less than 1% (Kinney, 1996a) (compare with fatty acid percentages presented in Table 2). Similar increases in oleic acid content have been achieved in rapeseed (Kinney, 1996a), cotton (Liu *et al.*, 2002) and *Arabidopsis* (Stoutjesdijk *et al.*, 2002) by suppression of FAD2 activity.

High stearic acid oils

Traditional breeding techniques have been employed for production of high stearic acid cultivars of soybean (Graef *et al.*, 1985) and sunflower (Osorio *et al.*, 1995), but lack of genetic variation and complications arising from genetic polyploidy have hampered production of high stearate lines in other crop plants. GE technology has been used successfully to overcome these genetic limitations in several crop plants, including canola (Hawkins and Kridl, 1998) and cotton (Liu *et al.*, 2002).

The primary approach for generating plant oils enriched in stearic acid has been to enhance metabolic flow from palmitic to stearic acid in developing seeds. As shown in Fig. 1, there are several enzyme targets that might be manipulated to achieve this goal, including suppression of FatB thioesterase activity (prevents cleavage of 16:0-ACP), increasing KASII activity (enhances conversion of 16:0-ACP to 18:0-ACP), increasing FatA thioesterase activity (promotes cleavage of 18:0 from ACP, thereby preventing desaturation by stearoyl-ACP desaturase), and/or suppressing stearoyl-ACP desaturase activity (blocks conversion of 18:0-ACP to 18:1-ACP). Each of these manipulations should favour production of stearic acid at the expense of palmitic acid, and suppress its further desaturation to oleoyl-ACP. The selection of a specific GE strategy, however, depends upon the relative flux of fatty acids through these different metabolic steps in the target plant.

Although a slight increase in stearic acid content has been obtained by overexpression of KASII in transgenic plants (Kinney, 1996b), a much larger increase in stearic acid has been achieved by increasing FatA thioesterase activity. For example, FatA activity was increased in canola by transgenic expression of the FatA thioesterase gene from mangosteen (*Garcinia mangostana*), a tropical fruit that accumulates high amounts of stearic acid (56%) (Padley *et al.*, 1994). The stearic acid content in the seed oil of the resulting transgenic canola plants increased from 2 to 22% (Hawkins and Kridl, 1998). Recently, the specific activity of the mangosteen FatA towards stearoyl-ACP was increased 13-fold by DNA site-directed mutagenesis, resulting in transgenic plants that accumulated up to 70% more stearate than plants expressing the wild-type enzyme (Facciotti *et al.*, 1999).

The highest stearic acid content, however, has been obtained by down-regulating stearoyl-ACP desaturase activity. For example, antisense suppression of stearoyl-ACP desaturase mRNA in either cotton or canola seeds results in an increase in stearic acid content from 2 to 40% (Knutzon *et al.*, 1992; Liu *et al.*, 2002).

Although production of oils with high stearic or oleic acid content has been very successful, it is noteworthy that alterations of seed oil fatty acid composition might compromise other agronomic traits. *Arabidopsis* seeds containing high oleic acid, for example, do not develop properly at low temperatures, resulting in lower total seed oil content and germination than wild-type seeds (Miquel and Browse, 1994). Cotton and canola engineered for high stearic acid content in seed oil exhibited poor germination and reduced survival of seedlings (Knutzon *et al.*, 1992; Liu *et al.*, 2002). These data suggest that care must be taken to ensure that performance in the field is not affected when the fatty acid composition is altered.

High palmitic acid oils

Despite certain health concerns associated with consumption of saturated fats, particularly animal fats, there is consistent demand for, and use of, plant oils containing high amounts of saturated fatty acids, as evidenced by the fact that palm oil (44% palmitate) is the second most abundant oil produced worldwide (Edem, 2002). Cocoa seed oil, referred to as cocoa butter, is another oil that contains high amounts of total saturates (25% palmitic acid) (Padley *et al.*, 1994). These oils have specialized uses in both edible and industrial applications, including bakery shortenings, confectionary products and soaps. The generation of high amounts of palmitic acid in oilseed crops, to replace similar oils derived from palm or cocoa, has been achieved by modulating enzyme activity in

favour of palmitic rather than stearic acid production. For example, palmitic acid content was increased in soybean oil from 10 to 50% by suppressing KASII activity and increasing FatB thioesterase activity (Kinney *et al.*, 1998).

Reduction of both palmitic and stearic acid content in oils

In the examples cited above, the metabolic pathways of the plastid were manipulated in favour of either palmitic or stearic acid production. In some cases, however, it is beneficial to reduce the content of both, e.g. in the production of oils with very low amounts of saturated fatty acids. Under normal metabolic conditions, the palmitic or stearic acids released from ACP in the plastids are considered as end products, since they are generally not desaturated. However, GE technology has provided a mechanism for desaturation of these fatty acids after they have exited the plastid. This strategy is based on the observation that yeast and mammalian cells contain Δ^9-desaturases located in the ER that act upon both palmitoyl- and stearoyl-CoA substrates. Expression of either yeast or mammalian Δ^9-desaturases in transgenic plants results in a modest reduction of saturated fatty acids and an increase in unsaturated fatty acid content (Polashock *et al.*, 1992; Grayburn and Hildebrand, 1995). Although these changes in fatty acid composition are most pronounced in leaf tissue, further improvements in seed-specific activity may provide an effective mechanism for producing ultra-low saturated vegetable oils (Moon *et al.*, 2000).

Oils enriched in medium-chain fatty acids

Mixtures of various vegetable oils can be chemically interesterified[2] to produce so-called structured TAGs that have unique chemical or physical properties not present in the natural oils. These TAGs are often used in specialized applications, such as medical and confectionary products (Fitch-Haumann, 1997). Most of these structured TAGs contain medium-chain fatty acids that are manufactured from fractionated oils derived from coconut and palm kernel (Broun *et al.*, 1999). Identification of the FatB thioesterases that mediate the accumulation of short-chain fatty acids in various seed oils, however, has provided a mechanism for production of medium-chain fatty acids in the TAGs of conventional oilseed crops. For example, the California Bay tree expresses a medium-chain-specific FatB thioesterase that is responsible for the accumulation in seed oil of approximately 60% lauric acid (12:0) (Davies *et al.*, 1991; Pollard *et al.*, 1991; Voelker *et al.*, 1992). Expression of this FatB thioesterase in transgenic rapeseed results in seed oil also containing up to 60% lauric acid (Voelker *et al.*, 1992, 1996). Stereochemical analysis revealed that lauric acid is present primarily at the *sn*-1 and *sn*-3 positions of glycerol, but is excluded from the *sn*-2 position, suggesting that the endogenous LPAT enzyme in rapeseed is unable to incorporate lauric acid at the *sn*-2 position. Subsequent transformation of this transgenic rapeseed with a gene encoding an LPAT from coconut, which does accumulate short-chain fatty acids at the *sn*-2 position, further increased the amount of lauric acid in the transgenic seed oil up to 67% of total fatty acids (Knutzon *et al.*, 1999).

Specialized FatB thioesterases have been cloned from species of the genus *Cuphea*, which contain up to 90% medium-chain fatty acids (8:0 and 10:0) in their seed oils (Graham *et al.*, 1981; Graham, 1989). Expression of these FatB thioesterase enzymes in transgenic canola results in significant accumulation of medium-chain fatty acids in seed oil, accounting for up to 40% of the total fatty acid composition (Dehesh *et al.*, 1996).

However, increasing medium-chain fatty acid content to exceptionally high levels by introducing specific FatB thioesterases is not without its challenges. For example, biochemical analyses of transgenic rapeseeds containing the highest amounts of lauric acid revealed an increase in the activity of enzymes responsible for degradation of medium-chain fatty acids (Eccleston and Ohlrogge, 1998), indicating that the (over)production of exotic fatty acids in transgenic plants stimulates metabolic pathways involved in their catabolism.

Oils containing very long chain polyunsaturated fatty acids

Very-long-chain polyunsaturated fatty acids, such as arachidonic acid (20:4), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), are important components of human nutrition that are typically obtained from fish oils or certain marine microalgae. In light of a declining and potentially unsustainable supply from fish, there is considerable interest in transferring appropriate genes for long-chain polyunsaturated fatty acid biosynthesis to oilseed crops (Domergue *et al.*, 2005; Singh *et al.*, 2005). Recently, genes for each of the biosynthetic steps have been cloned and characterized (Sayanova and Napier, 2004), and expression of these genes in linseed results in accumulation of minor amounts (~5%) of these fatty acids in seed oil

[2]Interesterification involves the interchange of fatty acids at different positions (*sn* – 1, *sn* – 2 and *sn*-3) on the glycerol backbone of TAGs.

(Abbadi *et al.*, 2004). Significantly higher amounts of these fatty acids have been produced in transgenic soybean (20% EPA, 3% DHA), suggesting that oilseed crops can serve as a renewable, sustainable source of these important fatty acids (Kinney *et al.*, Patent application WO 2004/071467 A2).

Production of oils with industrial potential

Unusual monoenoic fatty acids

The identification of enzymes responsible for the synthesis of unusual monounsaturated fatty acids (i.e. monoenes other than oleic acid) in non-traditional crop plants has provided an avenue for production of such fatty acids in transgenic oilseed crops. Some of these unusual monoenoic fatty acids, such as petroselinic acid and Δ^5-eicosenoic acid, have potential use in industry (see below). Unusual monoenes are synthesized by at least three different routes in plant cells, including the activity of diverged forms of plastidial stearoyl-ACP desaturases, elongation of either normal or unusual monoene fatty acid products, and/or the activity of ER-localized fatty acyl-CoA desaturases.

Examples of diverged plastidial desaturases cloned to date are Δ^9-16:0-ACP desaturases from cat's claw (*Doxantha unguis-cati*) (Cahoon *et al.*, 1998) and milkweed (*Asclepias syriaca*) (Cahoon *et al.*, 1997), Δ^9-14:0-ACP desaturase from geranium (*Pelargonium xhortorum*) (Schultz *et al.*, 1996), Δ^6-16:0-ACP desaturase from black-eyed Susan vine (*Thunbergia alata*) (Cahoon *et al.*, 1994) and a Δ^4-16:0-ACP desaturase from coriander (*Coriandrum sativum*) (Cahoon *et al.*, 1992). Coriander is a rich source of petroselinic acid ($18:1\Delta^{6cis}$), an isomer of oleic acid that is a solid at room temperature (unlike oleic acid, which is a liquid) and has a melting temperature of 33 °C (Broun *et al.*, 1999). These physicochemical attributes, coupled with the presumption that monounsaturated fatty acids have positive health benefits, initially made petroselinic acid an attractive candidate for solid fat substitutes. However, this potential was tempered by rat feeding studies, which demonstrated that it increased liver weight (Richter *et al.*, 1996) and had other undesirable physiological effects (Weber *et al.*, 1995).

The usage of petroselinic acid is now restricted to industry; chemical cleavage of the double bond at the Δ^6 position gives rise to lauric acid, a component of detergents and surfactants, and to adipic acid, which is the monomeric component of nylon 66 (Cahoon *et al.*, 1992). Recent attempts to increase (via GE) the amount of petroselinic acid in transgenic crop plants have resulted in only modest success. For instance, while petroselinic acid accounts for approximately 70% of coriander seed oil, expression of the coriander Δ^4-16:0-ACP desaturase in transgenic tobacco resulted in accumulation of only 4% petroselenic acid (Cahoon *et al.*, 1992). These results suggest that additional enzymes from coriander are likely required for its efficient production and accumulation in transgenic seeds.

The second mechanism of unusual monoene production is the elongation of either normal or unusual monounsaturated fatty acids in the plastid or extraplastidial compartments. For example, in many species of *Brassica* (e.g. rapeseed) or *Arabidopsis*, a portion of oleoyl-CoA exported from plastids is elongated, in a series of reactions similar to plastidial fatty acid biosynthesis, to produce erucic acid ($22:1\Delta^{13cis}$). Since the four additional carbon atoms are appended to the carboxyl end of oleoyl-CoA, the double bond that was initially at the Δ^9 position of oleic acid is located at the Δ^{13} position of the 22 carbon fatty acyl-CoA end product. Although breeding efforts effectively reduced erucic acid for production of a useful edible oil (canola), the presence of this fatty acid in oils has properties that are of value in certain non-food applications, and high erucic acid rapeseed is cultivated specifically for these industries.

The third mechanism of unusual monoene production involves a novel ER-bound fatty-acyl-CoA desaturase, which has been recently identified in meadowfoam (*Limnanthes alba*) (Cahoon *et al.*, 2000). Its oil is novel in that over 95% of the fatty acyl groups are longer than 18 carbons and about 90% of these fatty acids have double bonds in the Δ^5 position (Phillips *et al.*, 1971). The major fatty acid is $20:1\Delta^{5cis}$ (approximately 60%), a fatty acid with physical properties useful in formulations of cosmetics, surfactants and lubricants (Burg and Kleiman, 1991; Erhan *et al.*, 1993). The biosynthesis of $20:1\Delta^{5cis}$ is quite different from the synthesis of erucic acid ($22:1\Delta^{13cis}$), since palmitoyl-CoA (rather than oleoyl-CoA) is the substrate for fatty acid elongation, and a 20:0 serves as the substrate for Δ^5 desaturation (Pollard and Stumpf, 1980). Using a genomics-based approach, Cahoon *et al.* (2000) identified cDNAs encoding a novel elongase and mammalian/yeast-like fatty-acyl-CoA desaturase responsible for the synthesis of $20:1\Delta^{5cis}$ in meadowfoam. Co-expression of both enzymes in transgenic soybean increases accumulation of total Δ^5 fatty acids up to 12%, mostly in the form of $20:1\Delta^{5cis}$. These results highlight the importance and potential of transferring multiple enzymes from the source plant for efficient production of novel fatty acids in the transgenic host plant.

Exotic fatty acids containing additional functional groups

While the majority of oilseed crops contain some proportion of the common polyunsaturated fatty acids

that are present in cell membranes, seed oils of non-traditional crop plants may contain fatty acids with conjugated double bond systems, acetylenic (triple) bonds, or have additional functional moieties, such as hydroxy or epoxy groups (Smith, 1970). Many of these so-called 'exotic' fatty acids have potential uses as industrial raw materials (oleochemicals) that could serve as alternatives to traditional petrochemicals. For instance, castor bean (*Ricinus communis*) oil, which contains up to 90% ricinoleic acid (12-hydroxy-9-octadecenoic acid), is chemically modified industrially to produce a variety of products, including lubricants, polyamide 11 (Nylon 11), coatings, inks, sealants, surfactants, emulsifiers, encapsulants, plastic films and formulations of biodiesel.

Despite the commercial importance of castor oil, great care must be used during the cultivation of the castor bean, since its seed also produces a potent toxin (ricin) and allergenic proteins. Castor bean, therefore, is an excellent candidate for GE technology focused on elimination of the toxic components, or the transfer of castor oil biosynthetic genes to other non-toxic hosts (Auld *et al.*, 2001). Ricinoleic acid is synthesized by a hydroxylase enzyme that shares extensive amino acid similarity with FAD2 (van de Loo *et al.*, 1995). Identification and expression of the castor bean hydroxylase gene in transgenic *Arabidopsis* results in accumulation of up to 17% ricinoleic acid in seed oil (Broun and Somerville, 1997). Additional increases in ricinoleic acid in transgenic plants will likely require co-expression of castor bean fatty acyl-CoA ligase and DGAT genes, which catalyse preferential incorporation of ricinoleate into TAGs (McKeon *et al.*, 1999).

Diverged FAD2 enzymes have also been cloned from various plant species that accumulate epoxy, acetylenic and/or conjugated fatty acids (Shanklin and Cahoon, 1998). For example, genes responsible for epoxy and acetylenic fatty acid production have been isolated from small flowering plants of the genus *Crepis* (Lee *et al.*, 1998), as well as the moss *Ceratodon purpureus* (Sperling *et al.*, 2000), while genes for conjugated fatty acid biosynthesis have been isolated from ornamental flowers (*Impatiens balsamina*: Cahoon *et al.*, 1999; *Calendula officinalis*: Cahoon *et al.*, 2001; Qiu *et al.*, 2001), bittergourd (*Momordica charantia*) (Cahoon *et al.*, 1999) and the tung tree (*Aleurites fordii*) (Dyer *et al.*, 2002). Among these species, only the tung tree is commercially cultivated for production of its seed oil (tung oil), a high-value industrial drying oil. The other species do not have agronomic traits conducive to large-scale production; therefore, GE provides an avenue for production of their exotic fatty acids in more conventional oilseed crops. Overall, the expression of divergent FAD2 enzymes responsible for synthesis of epoxy, acetylenic or conjugated fatty acids in transgenic plants has generally met with modest success, with values of introduced fatty acids ranging from 15 to 30% (Thelen and Ohlrogge, 2002). As with castor bean hydroxylase, it is likely that additional enzymes from the specific source plant will be required for the efficient synthesis and accumulation of these exotic fatty acids in transgenic hosts (Voelker and Kinney, 2001).

While diverged FAD2s represent the majority of enzymes cloned to date, a number of other enzymes have shown promise for production of exotic fatty acids in transgenic plants. For example, a P450-type epoxygenase from the seeds of the spurge plant (*Euphorbia lagascae*) is capable of producing 13% vernolic acid (12-epoxyoctadeca-*cis*-9-enoic acid) in transgenic tobacco callus (Cahoon *et al.*, 2002), and a Δ^6-desaturase from borage produces approximately 20% Δ^6-containing fatty acids, including γ-linolenic acid ($18{:}3\Delta^{6cis,9cis,12cis}$) and stearidonic acid ($18{:}4\Delta^{6cis,9cis,12cis,15cis}$), in transgenic tobacco plants (Sayanova *et al.*, 1997).

One of the unexpected, and potentially challenging, problems encountered when (over)producing exotic fatty acids in transgenic plants has been the concomitant increase in oleic acid in the seed oil (Broun and Somerville, 1997; Broun *et al.*, 1998; Cahoon *et al.*, 1999; Singh *et al.*, 2001). This is likely due to suppression of endogenous FAD2 enzyme activity by the presence of the exotic fatty acid in ER membranes (Cahoon *et al.*, 2002). Interestingly, co-expression of FAD2 and epoxygenase genes from *Crepis palaestina* in transgenic *Arabidopsis* plants reduces the typical high-oleate phenotype, suggesting that *Crepis* FAD2 tolerates the presence of epoxy fatty acids better than the endogenous *Arabidopsis* FAD2 (Singh *et al.*, 2001).

Waxes

Waxes have unique physical properties that make them useful in many applications, including cosmetic formulations, food products and industrial lubricants. Traditional sources of waxes include the whaling industry, because a substantial portion of whale fat is composed of wax. However, due to restrictive legislation on whaling, there has been increased demand for alternative sources of waxes.

Plants such as the desert jojoba (*Simmondsia chinensis*) accumulate wax esters in their seeds, but do not possess sufficient agronomic characteristics for low-cost, large-scale wax production. Transfer of the wax biosynthetic pathway to higher-yielding oilseed crops is of great interest. Over the past several years, elucidation of the wax biosynthetic pathway has facilitated the cloning of genes involved, and initial attempts to modify wax content in seed oil have proven successful. For instance, co-expression of three

different wax biosynthetic genes in transgenic *Arabidopsis* results in seed oil composed of up to 70% wax esters (Lardizabal *et al.*, 2000). The transfer of this wax biosynthetic pathway to traditional oilseed crops may result in a renewable source of wax that could compete with, or eventually replace, petroleum-based or animal-derived products.

Increasing seed oil yields

Increasing the overall oil content in plant seeds will directly benefit commercial oilseed production, due to increased yields from a given area of crop acreage. Although the cellular and molecular factors regulating seed oil content are largely unknown (Roughan, 1997; Bao and Ohlrogge, 1999), there are several examples of increased crop seed oil yields produced via GE. For instance, expression of a yeast *sn*-2 acyltransferase (LPAT) in transgenic *Arabidopsis* or *Brassica napus* results in a significant increase in overall seed oil content in both plants, up to 48% of the total (Zou *et al.*, 1997). Similarly, overexpression of endogenous DGATs in *Arabidopsis* and tobacco promotes modest increases in seed oil content in both (Bouvier-Nave *et al.*, 2000). Although the mechanism(s) by which these acyltransferases enhance seed oil content are not known, the data indicate that seeds have the capacity to increase fatty acid biosynthesis in response to increased TAG production. In addition, enhancing the source of fatty acids by increasing their rates of biosynthesis also increases total seed oil content (Roesler *et al.*, 1997).

Perspectives and challenges

The rapid adoption of first generation GM crops, containing a variety of pest management traits, suggests that GE technology will continue to play an important role in shaping trends in oilseed markets. GE technology holds great promise for improving nutritional characteristics of seeds and seed oil, and expanding the uses and marketability of oilseed crops. Manipulating endogenous fatty acid ratios for improved nutrition or oil stability has proven to be readily accessible by GE technology, but production of exotic fatty acids in temperate oilseed crops has met with limited success. It will be critically important to elucidate the factors that limit the synthesis and accumulation of exotic fatty acids in transgenic plants (Suh *et al.*, 2002), since high production of these fatty acids will simplify their purification. This knowledge will likely be obtained by a combination of molecular genetic, genomic and cell biology oriented studies that shed light on the interrelationships of fatty acid biosynthesis, modification and storage within developing seeds. Successful production of oilseed crops, containing high amounts of exotic fatty acids in seed oil, will ultimately provide renewable resources of oleochemicals that can compete with, and eventually replace, some of the non-renewable petrochemicals derived from fossil fuels.

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